c) obtaining a primary cell population from said immunized human comprising T cells capable of secreting cytokines;

d) adding said primary cell population to said microwell [comprising a hydrophobic membrane having a first cytokine binding ligand,] under conditions such that said T cell secretes a cytokine that binds to said first cytokine binding ligand; and

e) detecting said secreted T cell cytokine.

Please add the following claims:

20. (New) The method of Claim 19, wherein said detecting of step (e) comprises use of a second cytokine binding ligand.

21. (New) The method of Claim 20, wherein said second cytokine binding ligand is conjugated to an enzyme.

REMARKS

Claims 1-2 and 18 are at issue and stand rejected. Claim 18 has been amended and Claims 20 and 21 are hereby added. For clarity, the Examiner's rejections are set forth below:

(1) Claim 1 is rejected under 35 U.S.C. § 103 as being obvious over Namikawa in view of Tobin and prior art disclosed in the specification (Alvord, Zamvil and Kimball).

(2) Claims 1, 2 and 18 are rejected under 35 U.S.C. § 103 as being obvious over Namikawa in view of Tobin and prior art disclosed in the specification (Alvord, Zamvil and Kimball) as applied to Claim 1, and further in view of Goodwin and Viselli.

Applicants believe that the following remarks traverse the Examiner's rejection of the claims. These remarks are presented in the same order as they appear above.

(1) Claim 1 Is Unobvious.

Claim 1 was rejected under 35 U.S.C. § 103 as being obvious over Namikawa in view of Tobin (and other references of record). Applicants disagree.

A. There is no basis for combining the cited art

Where is the Examiner's basis for combining the cited references? To establish a *prima facie* case of obviousness, "there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference teachings." M.P.E.P. § 2143. The Examiner has failed to establish a basis to combine the references in this case. Instead, the Examiner merely states that "one skilled in the art would have been motivated" (Office Action, page 2). This is not a **reason** - this is a *conclusion* with no basis. Applicants submit that the desirability of the invention cannot supply the motivation to combine the references as suggested by the Examiner, but instead there must be some suggestion or motivation in the references themselves that leads to such a combination. The Federal Circuit, in a recent decision, articulates this position, stating:

> To prevent the use of hindsight based on the invention to defeat patentability of the invention, this court requires the examiner to show a motivation to combine the references that create the case of obviousness. In other words, the examiner must show **reasons** that the skilled artisan, confronted with the same problems as the inventor and with no knowledge of the claimed invention, would select the elements from the cited prior art references for combination in the manner claimed.

See In re Rouffet et al., No. 97-1492, 1998 U.S. App. Lexis 16414, at 15-16 (Fed. Cir. July 15, 1998) (emphasis added). The Examiner does not 'show reasons' why one skilled in the art would be motivated to combine these reference, but instead merely argues that "there is a reasonable expectation of success" - once combined. This is error.

The Examiner is not free to combine and then argue there is a reasonable expectation of success. The Examiner must **first** establish a basis for the combination. Once a basis for combining the art is established, the Examiner can argue about the expectation of success. However, the Examiner cannot use an expectation of success - gained through hindsight - as an excuse to combine the art. Since the Examiner offers nothing here in terms of reasons, he has failed to satisfy this threshold requirement and the combination of the art cannot stand.

B. Namikawa Does Not Teach The Invention

The Examiner argues that "Namikawa *et al.* teach that immunization with MBP in IFA prevents EAE in rats." (Office Action, page 2). The Examiner also points to the sentence "Furthermore, immunization of rats by injection of BP in IFA not only prevents subsequent active or passive induction of EAE, but also has been reported to induce cells capable of preventing subsequent active sensitization of recipients." (Office Action, page 2). The Examiner, however, fails to take note that the above-quoted sentence is simply observing what *others* have reported ("has been reported").

The Namikawa paper itself is in some conflict with what others have reported. Specifically, the paper notes that "spleen cells from rats injected repeatedly with BP/IFA **transfer** EAE" (see the Abstract, p.932). Previously, some reports (e.g. Miller) had suggested BP/IFA-sensitized spleen cells were suppressive. The Namikawa group reports the opposite:

> "In contrast, we found not only that co-culture of BP/IFA and BP/CFA spleen cells with BP did not prevent the ability of BP/CFA spleen cells to transfer EAE, but also that BP/IFA spleen cells cultured alone with BP were capable of transferring acute EAE to the recipients."

The Examiner has apparently overlooked this point. For confirmation of the nature of the findings reported in the Namikawa paper, the Examiner is encouraged to read the Discussion section of the paper (p. 933, right-hand column), where it is noted that:

> "That acute EAE could be induced in recipients of BP/IFA spleen cells after culture with antigen was somewhat unexpected."

The Discussion goes on in an attempt to explain the results in view of previous contradictory reports.

What would one skilled in the art believe after reading the Namikawa paper? Clearly, the picture is complex. The BP/IFA treated spleen cells *are capable of transferring disease.* When transferred, they are **not** - at least according to Namikawa - protective (e.g. suppressive). Moreover, even in the case of the animals supplying the spleen cells for transfer, protective effects within these donor animals were not complete. The reference notes that "two of the nine rats pretreated with BP/IFA developed clinical signs of EAE after challenge." (p. 933, left-hand column, second sentence).

When one skilled in the art looks at the Namikawa paper as a whole, there are teachings which complicate any simply protective model. The Examiner is not free to ignore these teachings. When these teachings are considered, the reference does not teach in the direction of the present invention. Certainly, the authors do not conclude or advocate treatment of humans to protect against MS.

C. Tobin Cannot Be Used As A General Teaching

Given the deficiencies of the Namikawa reference, how does the Examiner build an argument that one skilled in the art would find the present invention obvious? In the present Office Action, the Examiner argues that the work of Namikawa makes the subject matter of Claim 1 obvious, because one skilled in the art "would have been motivated . . . because Tobin et al. teach treatment with autoimmune antigens for the treatment of human disease." Clearly, the Examiner is attempting to use Tobin as a general teaching. This argument by the Examiner is in direct contrast to previous arguments by the Examiner where the generality of the present invention was challenged and the negative results of Cohen were emphasized (*see* prior Office Action, page 3).

It is submitted that, even if the Tobin reference is (improperly combined), it adds nothing to the Examiner's argument. Tobin teaches nothing about MS. Tobin teaches GAD peptides for the treatment of insulin-dependent diabetes mellitus. The Examiner has pointed to nothing in Tobin that indicates the GAD methodology is applicable to other diseases. Therefore, Tobin cannot be used as a general teaching for an obviousness rejection against the present (amended) claims and the Examiner's attempt to extend the teachings of Namikawa with the teachings of Tobin fail.[1]

[1] As previously noted in a prior response, since Claim 1 has been amended to specify myelin basic protein, the teachings of Tobin are largely irrelevant.

D. The Other References Offer No Relevant Treatment Choices

The Examiner also cites references from the specification (e.g. Alvord), arguing that they teach "similarities between EAE and human MS." (Office Action, page 2). Applicants submit that these references offer no treatment choices that are relevant to the claimed subject matter of the present invention. It is submitted that the Examiner is simply attempting to piece together the invention and somehow bridge the gap between the primary references are the present invention.

The deficiencies in the primary references are not remedied by the Examiner's combination of art. Even if ALL of the references are combined (improperly), the Examiner can point to no teaching that human MS should be addressed in the manner of the present invention.

(2) Claims 1, 2 and 18 Are Not Obvious

Claims 1, 2 and 18 are rejected under 35 U.S.C. § 103 as being obvious over Namikawa in view of Tobin and prior art disclosed in the specification (Alvord, Zamvil and Kimball) as applied to Claim 1, and further in view of Goodwin and Viselli. Claims 2 and 8 are directed to cytokine testing. The Examiner notes that "Namikawa *et al.* teach that after immunization, the response of cells to a T cell mitogen is tested" (Office Action, page 4). Applicants agrees that the **proliferative** response to a T cell mitogen is tested. However, no cytokines are measured. In response, the Examiner argues "The response of T cells *would have been alternatively measured* using art known lymphokine assays" (Office Action, page 4) (emphasis added).

What does the Examiner mean when he says "would have been alternatively measured"? *Who* "would" have measured cytokines? *What* cited reference teaches the measurement of cytokines in combination with the method of Claim 1?

The Examiner appears to have simply concluded - with no basis - that one skilled in the art would combine cytokine testing with the method of Claim 1. This is unsupported speculation.

Moreover, the specific method for testing cytokines is not to be found. While Viselli teaches the use of PVDF membranes, the reference does not teach that the membranes should be precoated with a ligand that will bind the cytokine. When the full reference is reviewed (see Tab 1), it is clear that Viselli attaches cells to the membrane and the membrane binds the cytokine. Thus, even if combined (improperly), the cited art does not teach all of the limitations of Claim 18.[2]

The use of a hydrophobic membrane *with a ligand attached* has advantages (e.g. lower background) over the commonly used hydrophilic membranes. None of these advantages are disclosed in the art cited. Thus, Claims 2 and 18 should be allowed.

CONCLUSION

For the reasons set forth above, it is respectfully submitted that Applicant's claims as amended should be passed to allowance. Should the Examiner believe that a telephone interview would aid in the prosecution of this application, Applicants encourage the Examiner to call the undersigned collect at (617) 252-3353.

Dated: November 30, 1998

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[2] Claim 18 has been amended to create antecedent basis for "said microwell." The "precoated" language is explicitly supported in the specification at page 9, line 11.

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Detection and quantitation of interleukin-2 from individual cells *

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In this report we present the use of cell blotting for the detection of interleukin-2 (IL-2)-producing lymphocytes. This is a rapid and sensitive immunochemical method analogous to Western blotting of proteins. When combined with image analysis one can determine the percentages of IL-2 positive cells as well as quantitate the amount of IL-2 surrounding each cell. When bovine lymph node cells (bLNC) were stimulated with the combination of concanavalin A (ConA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 24 h, 46.4 ± 0.6 % stained positive for IL-2 and, on average, each cell produced 0.92 ± 0.6 pg of IL-2 in 24 h. Phytohemagglutinin (PHA) and TPA-stimulated human peripheral blood mononuclear cells (hPBMC) produced approximately the same amount, 0.86 ± 0.4 pg of IL-2 per cell in 24 h; 45.6 ± 3.6 % stained positive for IL-2.

Key words: Lymphocyte; Interleukin-2; Quantitation; Secretion; (Bovine); (Human)

Introduction

Stimulation of T lymphocytes by antigen or mitogenic lectins initiates a series of cellular events which results in proliferation. One such event is the secretion of the growth factor, IL-2 (reviewed by Smith, 1984, 1988). The association of IL-2 with the IL-2 receptor leads to division (Cantrell and Smith, 1983, 1985).

IL-2 activity in cell-free culture media is routinely measured by bioassays of IL-2-dependent cell lines (Gillis et al., 1978). More recently an immunoassay for IL-2 activity utilizing an antiserum against the IL-2 receptor was described (Igietesme and Herscowitz, 1988). With these methods one measures the net amount of secreted IL-2 remaining at the end of the incubation period. Not taken into account is the IL-2 that was produced but also consumed during the incubation. Furthermore, the number of IL-2 producing cells or the amount produced per cell can not be determined.

Quantitation of IL-2 secreted by individual T cells has not been explored extensively. In one study, the detection and quantitation of IL-2 produced by single human peripheral blood lymphocytes was determined by limiting dilution analysis after 3 days of incubation (Vie and Miller, 1986). Estimations at earlier times were not carried out. Rocha and Bandeira (1988) suggested the possibility of detecting the production of IL-2 from single cells of the mouse thymoma line, EL4, using limit-

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* Portions of this work were presented in abstract form at the Joint Meeting of ASCB and ASBMB in San Francisco, January 1989. J. Cell Biol. (1988) 107, 699a.

Abbreviations: bLNC, bovine lymph node cells; BSA, bovine serum albumin; CS, calf serum; ConA, concanavalin A; hPBMC, human peripheral blood mononuclear cells; IL-2, interleukin-2; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; RPMI, Roswell Park Memorial Institute medium 1640; ^{3}H-TdR, tritiated thymidine deoxyribose; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; 2ME, 2-mercaptoethanol.

ing dilution analysis. While a frequency of the number of IL-2-producing cells was given, determinations of the amount of IL-2 actually produced were not. Steinmann et al. (1983) demonstrated that IL-2-producing cells could be visualized by antibody to IL-2 followed by immunoperoxidase staining, but the method did not include quantitation of the secreted product.

In this report we present a technique for visualizing and quantitating IL-2 secreted from single cells. The method, cell blotting, is based on a procedure used to determine prolactin release from rat anterior pituitary cells (Kendall and Hymer, 1987). More recently it was used to quantitate interleukin-1 secretion from individual cells of the acute monocyte human leukemia line, THP-1 (Gaffney et al., 1988).

Briefly, the procedure involves the attachment of cells to a protein-binding membrane such as Immobilon PVDF (Millipore, Bedford, MA) and the absorption by that membrane of secretory products. The membranes are treated with primary antiserum directed against the secreted product, with enzyme-conjugated secondary antiserum and substrate to produce a color reaction. Cells surrounded by zones of secreted product are visualized microscopically. The amount of secreted product is determined using computerized image analysis. Both the intensity and area of secreted product are measured and compared to standards.

In the study of IL-2 secretion, cells used were bovine lymph node cells (bLNC) stimulated with the combination of ConA and TPA, human peripheral blood mononuclear cells (hPBMC) stimulated with the combination of PHA and TPA, and MLA 144 cells, a gibbon lymphosarcoma line which constitutively secretes IL-2 (Rabin et al., 1982). A determination of the number of IL-2-producing cells and of the amount of IL-2 secreted per cell was made.

Materials and methods

Cell culture

Bovine retropharyngeal lymph node cells, isolated as previously described (Mastro and Pepin, 1980), were cultured overnight at 1×10^7 cells/ml in Roswell Park Memorial Institute medium 1640 (RPMI) supplemented with 5% CS and 0.1% gentamycin sulfate. For IL-2 production, cells were resuspended to 5×10^6/ml, ConA (ICN Biologicals, Lisle, IL) added to 3 μg/ml final concentration, and TPA (Midland Chemical Corp., Brewster, NY) added to 60 ng/ml final concentration. The final concentration of the solvent for TPA, dimethylsulfoxide was 0.1%, a concentration shown previously not to affect proliferation (Mastro and Pepin, 1980). Heparinized human peripheral blood was collected and hPBMC separated by density gradient centrifugation with Histopaque 1077 (Sigma Chemical Co., St. Louis, MO). hPBMC were cultured for 2 h at 1×10^6 cells/ml in RPMI supplemented with 5% CS and 0.1% gentamycin sulfate. Differential staining of hPBMC following 2 h in culture revealed that fewer than 1% of the cells were polymorphonuclear cells, approximately 70% were lymphocytes and 30% were monocytes. To induce IL-2 secretion, PHA (Wellcome Reagents Division, Buroughs Wellcome Co., Research Triangle Park, NC) was added at 2 μg/ml and TPA at 20 ng/ml final concentrations. MLA 144 cells, a T cell line established from a gibbon ape lymphosarcoma which constitutively produces IL-2 (Rabin et al., 1982), were maintained in RPMI supplemented with 10% heat inactivated CS and 5×10^{-5} M 2ME. To measure IL-2, cells were seeded at 4×10^5 cells/ml in Dulbecco's modified Eagle's medium with 10% CS, 5×10^{-5} M 2ME and 10 ng/ml TPA (Aspinall and O'Gorman, 1987). Bovine IL-2-dependent cells, BT2 (obtained from Dr. Paul Baker, Immunex, Seattle, WA; and previously described (Picha and Baker, 1986)) were maintained at 5×10^5 cells/ml in RPMI supplemented with 10% CS, 0.1% gentamycin sulfate, 5×10^{-5} M 2ME, and 3.8 μg/ml ConA. Human recombinant IL-2 (DuPont Company, New England Nuclear Corp., Boston, MA) was added to 1 U/ml twice weekly. To remove bound IL-2, cells were suspended in 25 mM sodium acetate (pH 4) in serum-free RPMI for 1 min, diluted six-fold in RPMI, centrifuged (10 min, $200 \times g$), and resuspended in RPMI with 5% CS.

Cell blotting

A modification of the procedure described for prolactin-secreting pituitary cells (Kendall and

Hymer, 1987) was utilized. Unstimulated cells, cultured as indicated, were centrifuged (10 min, $200 \times g$), washed in serum-free RPMI, resuspended to 5×10^6 cells/ml in serum-free RPMI, stimulated with ConA and TPA (bLNC) or with PHA and TPA (hPBMC) to induce IL-2 production, and diluted with RPMI to 5×10^4 cells/ml. (Serum was inhibitory to cell attachment hence the use of serum-free medium.) Aliquots (5×10^3 cells in 100 μl) were placed in droplets on 1×1 cm squares of Immobilon transfer membrane that were glued to microscope slides with Household Goop (Eclectic Products, Carson, CA). The slides were placed in covered petri dishes lined with moist toweling and incubated in a humidified incubator (37°C, 5% CO_2) for various times to allow for cell attachment and IL-2 secretion. Petri dishes were removed from the incubator and the medium aspirated carefully from the membranes. To the area on the membranes from which the medium had been aspirated was added sequentially 125 μl droplets of the the following reagents: 0.05% glutaraldehyde in PBS, 30 min, room temperature; 0.15% phenylhydrazine hydrochloride (Sigma) in PBS, 30 min, room temperature; 1% BSA in PBS, 1 h, 37°C; primary antiserum, rabbit anti-IL-2 peptide (against residues 59–72, a gift from Dr. N.S. Magnuson, Washington State University, Pullman, WA and previously described (Weinberg et al., 1988)) 1/100 in 1% BSA in PBS, overnight, 37°C; secondary antiserum, horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) 1/100 in 1% BSA in PBS, 6 h, 37°C; substrate 0.05% 3,3′-diaminobenzidine (Sigma) in 0.01 M citrate buffer (pH 5.2), initiated with 4 μl 30% H_2O_2/ml citrate buffer, 1 h. Samples were washed twice with 125 μl volumes of PBS, added as droplets, between all steps. Following substrate incubation, squares were washed by flooding with distilled water and air dried. In other experiments the anti-IL-2 antibody was found to neutralize IL-2 activity in a bioassay. We also determined that extensive adsorption of the antiserum with freshly isolated unstimulated lymphocytes did not change its reactivity in the assay.

Quantitation

Secreted IL-2 was quantitated with an OASYS image analysis system (LeMont Scientific, State College, PA) which records grey levels between 1 and 255. Cells with secretions were focused at 400×, and manually outlined by the machine operator. In preliminary experiments we determined that outlining of the same cell and secretion several times by different operators or by the same operator all gave very similar values. Cells were digitized and removed from the images, leaving only the surrounding zones of secreted products. The average grey level of the outlined secretory zone as well as the area (mm^2) was measured by the instrument. The product of the average grey level and the area was defined as 'grey level units'.

In order to prepare a standard curve, purified human IL-2 (Electro Nucleonic, Silver Springs, MD) was serially diluted in RPMI, spotted (200 nl) onto Immobilon with a sequencing pipetman (Drummond Scientific, Broomall, PA), allowed to dry overnight, and treated with the same reagents as cell blots. Five replicates for each dilution were manually outlined and analyzed with the OASYS system. Because of the size of the standards, analyses were done at 40× magnification while individual cells were analyzed at 400×. We ascertained that analysis at 400× of small areas, comparable to the size of cells and within the larger area of standard spots, gave the same results as analysis of entire standard areas. A standard line was obtained by plotting the grey level units of the standards versus their concentrations on a log scale (see Fig. 2). A linear relationship was obtained between grey level units and IL-2 concentration between 0.0625 and 1.0 pg IL-2. Background grey levels for immunochemical-treated membrane with and without cells and for areas outside secretory zones on membranes with cells were determined to be very similar. Therefore an average background level was subtracted from the grey level units for standards and secretions. Values for IL-2 from cell secretions were obtained by directly reading from the standard curve.

In order to set uniform conditions of light in the image analysis system, transmitted light conditions were chosen so that both cells with secretions and IL-2 standards could be easily viewed and analyzed. The light of the microscope was adjusted for each magnification and for each field so that the exposure time on the system's Nikon

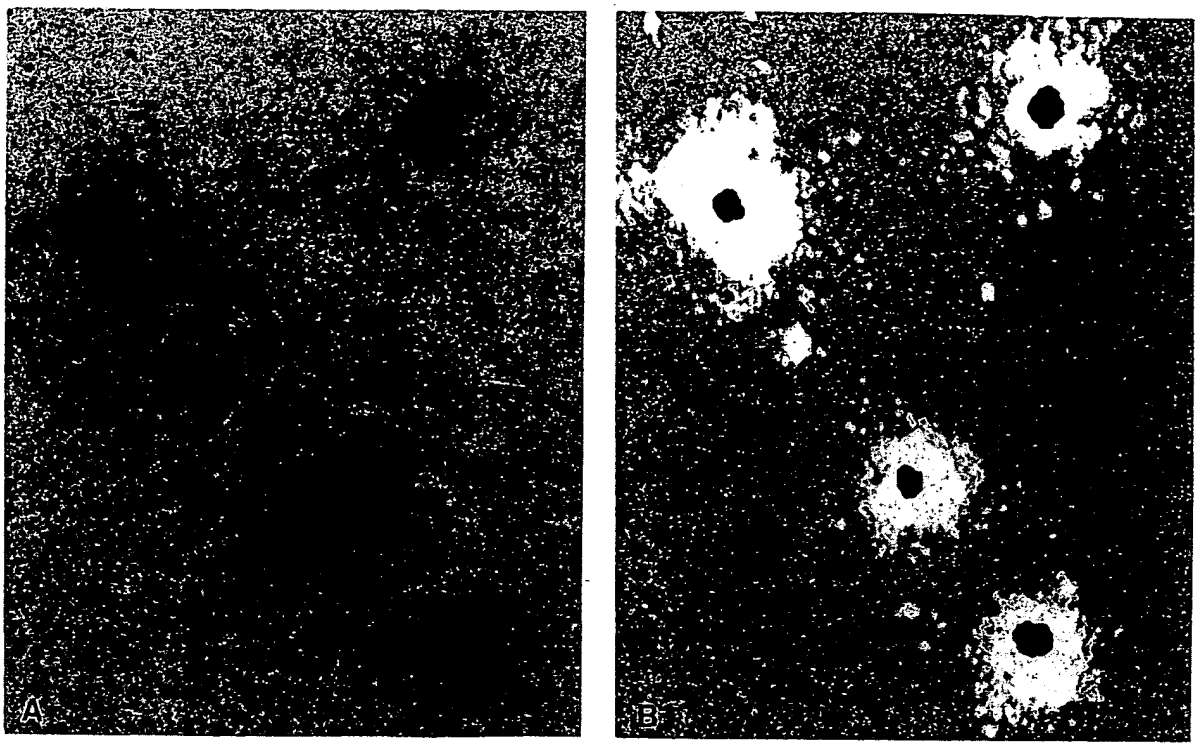

Fig. 1. Visualization of IL-2 secreted by bLNC. *A*: photomicrograph of a cell blot of bLNC incubated with 3 μg/ml ConA and 60 ng/ml TPA for 24 h. *B*: image enhancement of the same cell blot. Magnification is approximately 480×.

UFX II light meter display remained constant, ensuring that the absolute illumination was unchanged. These conditions were maintained throughout the study.

Determination of total cells and cells secreting IL-2

In order to determine the number of stained cells, cell blots were viewed microscopically (400 ×) and the anti-IL-2-reactive cells were counted. A minimum of 100 cells per sample was counted and the number of randomly chosen, contiguous microscope fields required to observe 100 cells recorded. In order to determine the total numbers of lymphocytes (stained and non-stained) present on the membranes, after image analysis the membranes were hematoxylin stained with Leukostat (Fisher Scientific, Orangeburg, NY) for 5 s each in each of the three reagents provided, according to manufacturer's instructions. Using this procedure it was seen that the cells remained intact for the duration of the cell blot procedure. To determine total numbers of bound lymphocytes, a minimum of 100 hematoxylin-stained cells per sample was counted and the number of fields necessary to view at least 100 cells was recorded. Hematoxylin staining obscured diaminobenzidine staining; therefore, cells stained by the cell blot procedure and total cells stained with hematoxylin were counted separately. To obtain percentages of anti-IL-2-reactive cells in each sample, initial counts of cell blots after staining for IL-2 were compared to the same hematoxylin-stained cell blots. The numbers were adjusted to standardize for the number of fields viewed.

Results

Visualization of secreted IL-2

Microscopic inspection of cell blots revealed that IL-2 secreted by individual cells was easily visualized (Fig. 1). For example, when bLNC which had been stimulated with ConA and TPA for 24 h were viewed microscopically, the stained,

TABLE I

SPECIFICITY OF IMMUNOREACTION FOR IL-2

Bovine LNC, MLA 144 cells, non-lymphoid cells, 3T3 fibroblasts, and BT2 cells, incubated in duplicate for 24 h, were treated with either rabbit anti-IL-2 antiserum or normal rabbit serum (NRS) (primary antiserum) followed by incubation with goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) or with normal goat serum (NGS) (secondary antiserum) as indicated. Unstimulated bLNC or bLNC stimulated with the combination of ConA and TPA were compared. BT2 cells, an IL-2-dependent bovine T cell line that can respond to IL-2 but can not make IL-2 were tested after incubation in the presence of IL-2. In one case ($BT2^a$) the cells were tested immediately. In the second ($BT2^b$) they were washed in sodium acetate to remove bound IL-2 (see materials and methods section). The number of stimulated cells which reacted with the cell blot reagents (+ + +) was two-fold greater than the number in the unstimulated samples (+), as described in Fig. 4. $BT2^a$ cells stained with no surrounding secretions (+). − indicates no positive secretions.

Antisera		Cultures		Reaction
Primary	Secondary	Cells	Additions	
Anti-IL-2	Anti-rabbit HRP	bLNC	None	+
Anti-IL-2	Anti-rabbit HRP	bLNC	ConA + TPA	+ + +
NRS	Anti-rabbit HRP	bLNC	ConA + TPA	−
Anti-IL-2	NGS	bLNC	ConA + TPA	−
NRS	NGS	bLNC	ConA + TPA	−
Anti-IL-2	Anti-rabbit HRP	none	ConA + TPA	−
Anti-IL-2	Anti-rabbit HRP	MLA 144	None	+ + +
Anti-IL-2	Anti-rabbit HRP	NIH 3T3	None	−
Anti-IL-2	Anti-rabbit HRP	$BT2^a$	None	+
Anti-IL-2	Anti-rabbit HRP	$BT2^b$	None	−

[a] Tested directly after incubation with IL-2.
[b] Washed in sodium acetate before testing.

secreted product was clearly visible around cells (Fig. 1*A*). In addition to the secretion, the cells themselves appeared to be darkly stained. However, through the use of image enhancement, secreted products were readily distinguished from cells and from background (Fig. 1*B*). Cells stimulated with ConA alone also produced IL-2 detected by cell blotting (data not shown).

When incubations were stopped earlier than 24 h, the intensity of staining was much less and stained product was visible around fewer cells. Upon microscopic inspection, only a small percentage of bLNC which were stimulated for 3 h stained and then only lightly with diffuse boundaries. Therefore, 24 h was chosen as the standard incubation time.

Cell blots of hPBMC appeared much the same as those of bLNC. After 3 h some cells were lightly stained and had secretions with low staining intensity; following a 24 h stimulation many cells were surrounded by darkly stained secretions (not shown).

MLA 144 cells looked somewhat different. While essentially all cells were stained, the intensity of the staining was lower, background staining higher, and the pattern of staining more diffuse than with primary lymphocytes (data not

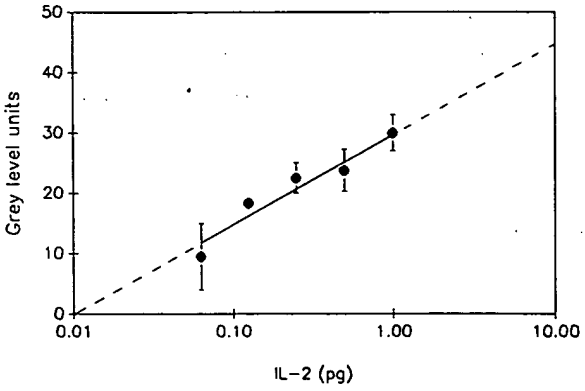

Fig. 2. IL-2 standard curve. Purified human IL-2 was diluted serially and 200 nl spotted onto membranes with five replicates per dilution. After immunochemical staining, the spots of reactivity were visualized, image analyzed and grey level units determined by multiplying the average grey level by the area of standard, as described in the materials and methods section. Background grey level units were subtracted from standard grey level units. Plotted are the averages of the replicates ± standard deviations.

shown). Also, because cells clumped together on the membranes as they do in suspension culture, there were few single cells with well-defined secretions making it difficult to accurately determine the secreted products.

Quantitation of IL-2 produced by individual cells

The amount of IL-2 secreted per cell was determined for the cells which had a clearly visible area of secretion. Comparison of cell products with a standard curve (Fig. 2) allowed quantitation of IL-2 secreted by individual cells. It was ascertained that cells were surrounded by stained secreted product only when IL-2-producing cells and specific primary and secondary antisera were used (Table I). When cells not known to secrete IL-2 (NIH 3T3 fibroblasts) were used or when no cells were used in the assay, neither stained cells nor secreted product was visible. Replacement of either or both primary and secondary antisera with pre-immune sera also prevented the visualization of IL-2-secreting cells. However, use of pre-immune serum led to labeling of an average 8% of lymphocytes alone, i.e., with no secretion. This labeling did not appear to be due to Fc receptor binding because it was not eliminated by including 1% non-immune goat serum in all of the incubation buffers. This labeling accounted for almost all positive cells without secretions.

Analysis of 50 bLNC incubated for 24 h with ConA and TPA revealed that the amount of IL-2 produced per cell ranged from 0.12 pg to 3.5 pg. On average, cells secreted 0.92 ± 0.6 pg of IL-2 per cell during that period (Fig. 3*A*). 50% of the total stimulated bLNC produced between 0.12 and 1.3 pg per cell and the remaining 50% of the total bLNC produced between 1.3 and 3.5 pg of IL-2 per cell in 24 h. Cell blots of bLNC from three other animals, in three separate experiments were also analyzed; in each case the values were found to fall within the same range. IL-2 produced by individual unstimulated bLNC, incubated for 24 h, was also detected. Fewer unstimulated cells produced IL-2 (see Fig. 4) and while the amount of IL-2 fell within the same range as stimulated bLNC, it averaged less than half (0.41 ± 0.07 pg/cell). Considering both the percentages of IL-2-secreting cells and the amounts secreted per cell, unstimulated cells produced approximately 17% of the IL-2 produced by cells stimulated with ConA and TPA.

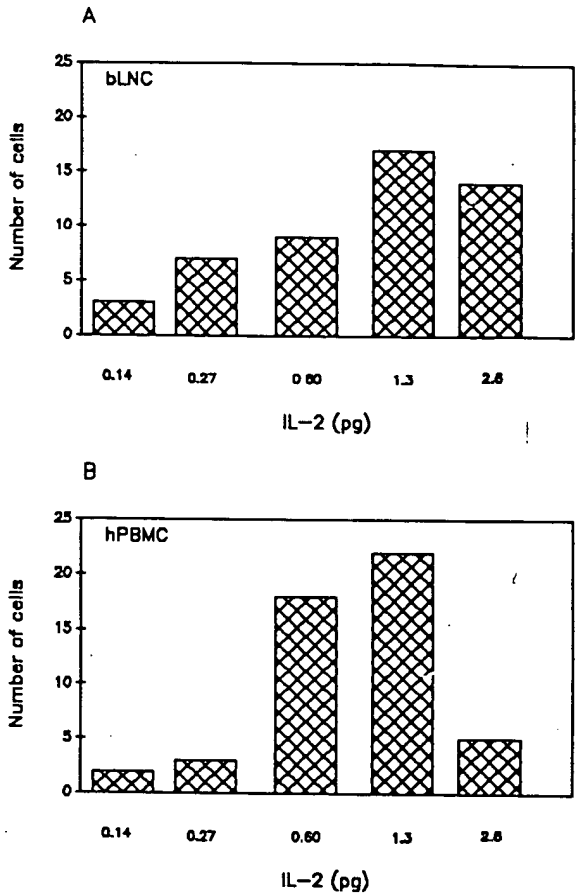

Fig. 3. IL-2 secreted by individual bLNC and hPBMC. *A*: bovine LNC were stimulated with ConA and TPA for 24 h before being incubated with anti-IL-2 antibody, peroxidase-conjugated antibody and diaminobenzidine substrate, as described in the materials and methods section. The amount of IL-2 produced by 50 cells was calculated from image analysis and compared with a standard curve. The average secretion per cell was 0.92 ± 0.6 pg. Cells from three other animals, in three separate experiments, were also analyzed for IL-2 production. In each case, values for IL-2 produced per cell fell within the same range. The number of cells producing IL-2 is plotted versus median pg of IL-2 per cell within each range. *B*: human PBMC stimulated with the combination of PHA and TPA were treated as described for bLNC. The secretions of 50 hPBL were measured. The average secretion per cell was 0.86 ± 0.4 pg. Two separate experiments using blood obtained from two normal donors on two different occasions were performed with similar results. As in *A*, numbers of cells producing IL-2 per cell within each range are plotted against median pg IL-2 per cell.

The IL-2 produced by 50 stimulated hPBMC

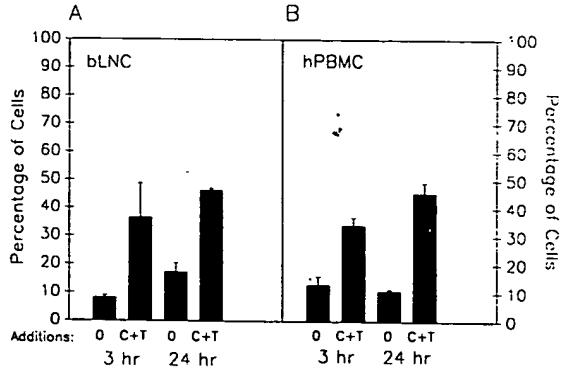

Fig. 4. Percentages of IL-2-secreting cells. *A*: bovine LNC, unstimulated or stimulated with ConA and TPA, and incubated for either 3 h or 24 h, were reacted with anti-IL-2 antiserum, and treated as in the legend to Fig. 3. Cells with secreted IL-2 were counted as described. Membranes were later stained with hematoxylin and eosin and the total numbers of cells seen after the immunochemical stain compared with the number of hematoxylin and eosin stained cells. Percentages of anti-IL-2 reactive cells, obtained by comparing numbers of cells on cell blots with total numbers of cells, are shown. Cells incubated for 3 h stained much less intensely than did those incubated for 24 h. Shown are the averages from duplicate samples of cells from three different animals evaluated in three separate experiments. *B*: human PBMC, unstimulated or stimulated for 3 or 24 h with PHA and TPA as described, were treated as the bLNC. Cells were counted after each staining procedure and the percentages which secreted IL-2 were determined. Two separate experiments were conducted using blood obtained from two normal donors on separate occasions. Shown are the averages ± standard deviations. C, ConA; T, TPA; P, PHA; 0, none.

during a 24 h incubation was also determined (Fig. 3*B*) and found to average 0.86 ± 0.4 pg/cell. 50% of the cells produced between 0.10 and 0.8 pg of IL-2/cell while the remaining 50% produced between 0.8 and 3.0 pg/cell in 24 h. This experiment was done twice with cells from two donors on 2 different days. Results from the second experiment were comparable to the first.

Quantitation of secretions from bLNC and hPBMC after 3 h incubation was not done since the boundaries of the secretions, present around a small percentage of cells, could not be determined accurately.

Percentages of anti-IL-2-reactive cells

As described in the materials and methods section, percentages of anti-IL-2-reactive cells were obtained by first counting the cells visible on membranes after immunoblotting and comparing these numbers with the number of cells counted on the same membranes after a second stain with hematoxylin. For bLNC, duplicate samples from each of three animals, in three separate experiments, were counted and for hPBMC triplicate samples from each of two normal donors on two separate occasions and in two separate experiments were counted (Fig. 4). After 3 h incubation 8% of unstimulated bLNC had visibly secreted IL-2. This number may represent cells activated in vivo. However, 37% of the bLNC that were stimulated with the combination of ConA and TPA for 3 h were anti-IL-2-reactive. After 24 h incubation on the membranes, 17% of unstimulated bLNC were anti-IL-2-reactive compared with 46% of stimulated bLNC. It should be noted that while some cells that were incubated for 3 h showed secreted products, the intensity of staining was much less than for those incubated for 24 h. The boundaries of the zones of secretions after 3 h were too diffuse to quantitate. Similar results were obtained with cell blots of hPBMC (Fig. 4*B*). In all cases, the numbers of anti-IL-2-reactive cells in the stimulated groups were several fold greater than those in the unstimulated groups (Fig. 4).

Of the anti-IL-2 reactive bLNC incubated for 24 h, with the combination of ConA and TPA, approximately 16% stained darkly with no visible secreted product. Because this value was about double the background number of cells which stained with pre-immune serum we thought that some stained cells may have bound exogenous IL-2.

In order to determine the appearance of cells which bound but did not make IL-2, a cell blot assay was conducted using BT2 cells, an IL-2-dependent line grown in the presence of IL-2. These cells, which are incapable of secreting IL-2, stained darkly after a 6 h incubation. However, when these cells were subjected to a brief acid wash to remove bound IL-2 before cell blotting, they did not stain (Table I). When bLNC were subjected to an acid wash prior to cell blotting, their appearance did not change; darkly stained cells with and without secretions were visible on membranes after incubations in cell blot reagents. The continued presence of cell-produced IL-2 throughout

the assay with bLNC may partially explain this result.

Discussion

Cell blotting proved to be a a useful technique for comparing the relative amounts of IL-2 produced by individual cells under given conditions of stimulation. Cells with secreted IL-2 was readily visible. IL-2 was detected following a 24 h incubation of cells with ConA and TPA or with ConA alone. When cells were stimulated for only 3 h, the intensity of staining was weaker as was the amount of secreted product. Since IL-2 accumulates during an incubation, one would expect less total IL-2 after 3 h than after 24 h.

For cell blotting, serum inhibited cell attachment to Immobilon membranes and also increased background staining. Hence, serum-free medium was used. However, because bLNC were cultured overnight and hPBMC were cultured for 2 h in the presence of 5% CS before they were washed and resuspended in serum-free medium, it is likely that some serum was still present in the cultures at the time of the addition of co-mitogens. Moreover, it has been reported by others that stimulation of rat splenocytes in serum-free and in serum-containing medium resulted in identical IL-2 titers (Valyakina et al., 1984); and, in a recent report of IL-2 production by bovine lymph node cells (Weinberg et al., 1988), serum-free conditions were employed.

One consideration in quantitating the assay was whether or not to include the areas of the cells themselves. Even though microscopically the cells appeared to have stained more intensively than the secretions, there is no evidence that this darker appearance was due solely to IL-2 on the cell surface. We chose not to include cells in quantitation for two reasons. First, there is a geometry and substrate concern, i.e., substrate deposited on the plasma membranes of round cells may not be equal to substrate deposited on flat Immobilon membranes. Second, deposition of stain appeared to be more intense around the periphery of the attached cells; therefore, visual inspection did not give an accurate estimation of the stained cell size. Because the cells were relatively uniform in size and the intensity of cell staining was approximately equal, the cell stain was considered a constant factor for the purposes of comparing relative amounts of secretion. Nevertheless, if we assumed that secretion over the cell to be of the same density as on the Immobilon membrane, the calculated increase in IL-2 secreted per cell was 5% at most. Moreover, it should be remembered that bioassays also only measure soluble and not cell-bound IL-2.

Bioassays of IL-2-dependent cell lines give a more general measure of IL-2 than the cell blot. While bioassays can be used to determine whether or not IL-2 has been produced under conditions of interest, the number of producing cells is not known. Moreover, bioassays most certainly underestimate the total amount of IL-2 because cell-produced IL-2 is consumed. Other factors which influence the results of bioassays include sensitivities of IL-2-dependent cells, and the presence of inhibitors or of other reactive growth factors such as IL-4. These factors did not appear to play a role in the cell blot assay. In contrast, with cell blotting we calculated the IL-2 produced per cell and found that on average, stimulated bLNC secreted 0.92 ± 0.60 pg (mean $\pm$ standard deviation) of IL-2 per cell in 24 h and hPBMC secreted on average 0.86 ± 0.4 pg per cell in 24 h. Vie and Miller (1986) reported that individual human cells stimulated with PHA produced a median amount of 0.92 pg of IL-2 per cell after 3 days, as determined by bioassay in a limiting dilution analysis. In their assay, 16.3% of the cells produced detectable levels of IL-2. Rocha and Bandeira (1988) also using limiting dilution analysis, reported that one out of 13, 6-thioguanine-resistant EL4 cell produced IL-2 in 48 h; They did not quantitate the amounts of IL-2.

While our estimates of the amounts of IL-2 produced per cell are similar to those of Vie and Miller (1986), the percentages of IL-2-producing cells detected by cell blotting were much higher than those reported by Vie and Miller (1986) or by Rocha and Bandiera (1988). Thus the question of differences in assay sensitivity levels arise. In the cell blot assay under the stated conditions, the lower limit of quantitation for secreted IL-2 was approximately 0.10 pg per cell while the lowest amount reported by Vie and Miller (1986) was

0.75 pg. This variation may result from the numbers of cells analyzed but also from differences in sensitivities of immunoassays and bioassays to IL-2. For example, when culture medium from stimulated hPBMC was tested immunochemically, a dilution of 1/256 was detected while a bioassay was sensitive only to a dilution of 1/32. In summary, there are many possibilities for variations between the assays carried out by different methods in different laboratories.

From analysis of cell blots we determined numbers of IL-2-secreting cells. In all cases, these numbers were at least two-fold and up to four-fold greater in co-mitogen-stimulated bLNC and hPBMC cultures than in unstimulated cultures. Approximately 8% of unstimulated bLNC and 13% of unstimulated hPBMC secreted IL-2 after 3 h incubation, and approximately 17% of unstimulated bLNC and 11% of unstimulated hPBMC secreted IL-2 after 24 h. For comparison, in a bioassay unstimulated culture medium had 5–10% of the activity of that of the stimulated (data not shown). Although we were surprised to see that greater than 10% of unstimulated cells stained with antisera to IL-2, this staining appeared to be specific for IL-2-producing lymphocytes as neither NIH 3T3 fibroblasts nor acid-washed bovine cytotoxic T cells, incapable of producing IL-2, stained. In searching the literature we found no other reports of immunochemical assay of IL-2 in unstimulated individual cells. In fact, in the first report of the serological visualization of IL-2, Steinmann et al. (1983) used a Jurkat cell line stimulated with PHA and TPA. Unstimulated cells were not used. Also, when cell blotting was used to quantitate IL-1 (Gaffney et al., 1988), approximately 60% of untreated THP-1 cells secreted IL-1 even though IL-1 activity was not detected in bioassays. Perhaps immunologically active but biologically unreactive lymphokines are present in unstimulated culture medium. It is certainly known that immuno- and bioactivity can vary greatly for many other biological materials, such as prolactin (Shah and Hymer, 1989).

While the percentages we obtained for IL-2-secreting cells are higher than those reported by others (Vie and Miller, 1986; Rocha and Bandeira, 1988), there are reports which suggest that IL-2 is produced by a large percentage of T cells from different subpopulations. For example, Zelarney and Belden (1986) reported that three subpopulations of total bovine peripheral blood T cells all produced IL-2 within 24 h after stimulation with ConA.

In summary, cell blotting is a rapid and sensitive means for detecting and comparing secreted IL-2 from individual cells. Determinations of percentages of IL-2-secreting cells and of relative amounts of IL-2 produced per cell under different conditions should help to elucidate further the roles of IL-2 in T cell biology and may have diagnostic value in comparing IL-2 release from T cells in a pathological state with normal cells.

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